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In a 2x2 factorial designed experiment involving chlorobenzene and γ-hexachlorocyclohexane (lindane), the hepatotoxicity induced by a challenge dose of chlorobenzene was altered by the pretreatments due to selective changes in various metabolic pathways. These changes resulted in either (1) a marginally significant decrease in hepatotoxicity (chlorobenzene-pretreatment); (2) significant reduction in both the incidence and severity of the lesions (lindane-pretreatment); or (3) absence of centrilobular hepatocellular necrosis in all but 1 of 12 rats where a minimal degree of necrosis was present (chlorobenzene + lindane-pretreatment).

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Antagonism of Chlorobenzene-Induced Hepatotoxicity by Lindane

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In two complete replicates of a 2 × 2-fractorial-designed experiment involving chlorobenzene and γ-hexachlorocyclohexane (lindane), the hepatotoxicity induced by a challenge dose of chlorobenzene was altered by the pretreatments due to selective changes in various metabolic pathways. Pretreatment with either toxicant, alone or in combination, elevated the relative metabolism of 1.12 g chlorobenzene/kg to conjugated and polar metabolites. The relative importance of these pathways was increased most by pretreatment with chlorobenzene + lindane and least with chlorobenzene. The incidence and severity of chlorobenzene-induced hepatocellular necrosis was dependent on how much the pretreatments increased excretion of these metabolites relative to that of *p*-chlorophenol, since the conjugates and polar metabolites represent an inactivation of the toxic chlorobenzene-3,4-epoxide whereas *p*-chlorophenol reflects its formation. Thus these changes in the metabolic pathways resulted in either (i) a marginally significant decrease in hepatotoxicity (chlorobenzene pretreatment); (ii) significant reduction in both the incidence and severity of the lesions (lindane pretreatment); or (iii) absence of centrilobular hepatocellular necrosis in all but 1 of 12 rats where a minimal degree of necrosis was present (chlorobenzene + lindane pretreatment). In this study, the effect of pretreatment with xenobiotics on chlorobenzene-induced hepatotoxicity was dependent on how much the pretreatments altered the inactivation of chlorobenzene-3,4-epoxide relative to its formation.

INTRODUCTION

Hepatic necrosis produced by halogenated aromatic hydrocarbons, such as chlorobenzene, is presumably due to covalent binding of an active intermediate to liver macromolecules (1–4). The initial step in the metabolism of chlorobenzene (Fig. 1) is thought to be the enzymatic insertion of oxygen into the aromatic nucleus to give the toxic, chemically reactive, chlorobenzene-3,4-epoxide (1, 5–8), the nontoxic chlorobenzene-2,3-epoxide (7–9), and the nontoxic *m*-chlorophenol (8). While most of the chlorobenzene epoxides are converted to a mercapturic acid by glutathione transferase (1, 2, 7, 10), the chlorobenzene-3,4-epoxide and the chlorobenzene-2,3-epoxide are rearranged nonenzymatically to *p*- and *o*-chlorophenol, respectively (8). The epoxides are also converted by epoxide hydrase to the corresponding dihydrodiols and then by dehydrogenation to the corresponding chlorocatechols (2, 10).

When a chemical is metabolized via multiple pathways, the effect of pretreatment with enzyme-inducing agents on the formation of a toxic metabolite depends on the subsequent relative activity of the pathways involved. For example, induction by phenobarbital markedly increases metabolism of bromobenzene to bromobenzene-3,4-epoxide and potentiates the concomitant hepatic necrosis (6). On the other hand, induction with 3-methylcholanthrene does not increase hepatic necrosis, presumably since enhanced metabolism proceeds via the nontoxic bromobenzene-2,3-epoxide (7, 9). Pretreatment with other xenobiotics such as the organochlorine insecticide γ-hexachlorocyclohexane (lindane), and chlorobenzene itself might also be expected to alter the hepatotoxicity of chlorobenzene. Both chlorobenzene (3, 11, 12) and lindane (13–17) induce hepatic en-

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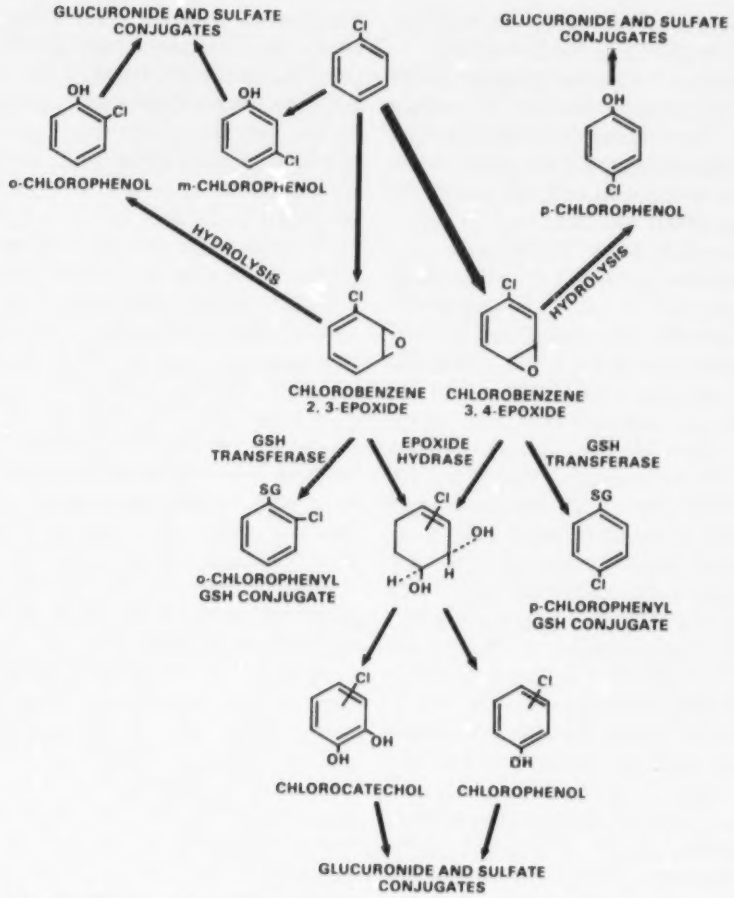

FIG. 1. *Mammalian metabolism of chlorobenzene to phenols, dihydrodiols, catechols, and glutathione conjugates.*

zymes, both compete for metabolic pathways such as glutathione transferase (18, 19), and traces of both may occur in industrial waste since lindane is synthesized by the direct action of chlorine on benzene in the presence in light (20). Thus synergism of chlorobenzene-induced toxicity, following pretreatment with either lindane or chlorobenzene, was anticipated. However, some preliminary dose–response experiments indicated that pretreatment with nontoxic levels of either lindane or chlorobenzene did not increase chlorobenzene-induced hepatotoxicity and mayeven have reduced it. Consequently, this study was designed to determine if pretreatment with either toxicant, alone or in combination, would alter the metabolism and/or hepatotoxicity of chlorobenzene.

METHODS

Chlorobenzene (99%) was obtained from Aldrich Chemical Company Inc., Milwaukee, Wisconsin. [U-^{14}C]Chlorobenzene (sp act 10 mCi/mmol, radioactive purity 96%) was obtained from Amersham, Arlington Heights, Illinois. Lindane (>99% γ-hexachlorocyclohexane) was obtained from the Environmental Protection Agency, Health Effects Research Laboratory, Ana-

lytical Reference Standards Repository, Research Triangle Park, North Carolina.

Thirty female, weanling Sprague–Dawley rats, individually housed in metabolism cages, were randomly assigned to one of five treatment groups. One group of six animals was dosed with only the peanut oil vehicle throughout the study and served as the negative controls for the histopathological examination (group D). The remaining animals were part of a 2 × 2-factorial-designed experiment involving lindane and chlorobenzene. For 7 days, the rats in the 2 × 2-factorial-designed experiment received daily po injections of peanut oil solutions of either 300 mg chlorobenzene/kg (group A), 20 mg lindane/kg (group B), 300 mg chlorobenzene + 20 mg lindane/kg (group AB), or unadulturated peanut oil (group C). On Day 8, all animals in the 2 × 2-factorial-designed experiment were dosed with 1.12 g chlorobenzene (containing 18.7 μCi [U-^{14}C]chlorobenzene) + 22.4 mg lindane/kg. Lindane was included in the challenge dose of chlorobenzene so that the effect of the treatment regimen on lindane metabolism could also be determined. This data will be presented in a separate report. Preliminary studies indicated that a single dose of lindane at this level did not affect the chlorobenzene-induced hepatotoxicity. The pretreatment level of chlorobenzene used was less than the nontoxic 400-mg/kg dose employed by Carlson and Tardiff to study the effect of chlorobenzene on the metabolism of foreign organic compounds (11). The pretreatment level of lindane is about 1/5 the LD_{50} (21) and has not produced overt signs of toxicity when previously employed in this laboratory. The challenge dose of chlorobenzene was based on the 10 mmol/kg chlorobenzene used by Oesch and co-workers to produce centrilobular hepatocellular necrosis in rats (3). Following treatment on Day 8, the animals were transferred to animal-containment chambers (Plas-Labs, Lansing, Michigan) and urine, feces, and expired air were collected for 24 hr. The animals were then transferred back to their former cages where they remained until sacrifice 24 hr later. Urine, feces, expired air, fat, liver, and lung samples were analyzed for radioactive content. Urine was extracted and partitioned as previously described (22). Liver, fat, lung, and feces samples were subjected to combustion in a Packard Tri-Carb Model 306 sample oxidizer and the radioactivity was determined in a Packard Tri-Carb Model 3380 liquid scintillation spectrometer employing a quench correction curve. At necropsy, liver samples were taken for histopathological examination. These were fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined by light microscopy. Representative sections were stained for glycogen (periodic acid–Schiff stain). In addition, frozen sections of some of the formalin-fixed tissues were stained for fat (oil red O or Sudan IV stains).

To verify the histopathological observations of the initial study and to further investigate the effect of the treatment on hepatic glutathione content and the biotransformation of chlorobenzene to *o*-, *m*-, and *p*-chlorophenol, two further experiments were conducted. In one, the experimental conditions, dose levels, and duration of the experiment were similar to the previous study except that unlabeled chlorobenzene was used on Day 8. In addition to repeating the histopathological examination of the liver, 24-hr urine samples were acidified and extracted twice with equal volumes of ethyl acetate to recover free *o*-, *m*-, and *p*-chlorophenol. The residual urine was acidified with HCl and hydrolyzed in an autoclave for 15 min under 15 lbs pressure and extracted twice with ethyl acetate to recover conjugated *o*-, *m*-, and *p*-chlorophenol. The chlorophenols were analyzed by reverse-phase high-pressure liquid chromatography on a 4.6 mm i.d. × 24 cm Zorbax ODS column (Dupont Company, Wilmington, Delaware) in 15 min using 1:43:56 acetic acid:acetonitrile:water at 0.3

ml/min. Quantification was performed by measuring UV absorbance at 277 nm. Upon sacrifice, 48 hr after receiving the challenge dose of chlorobenzene, liver samples were taken for analysis of reduced glutathione content by the method of Sedlak and Lindsay (24). In the final experiment, hepatic-reduced glutathione content was determined 5 hr after control rats, and rats treated 7 days with 20 mg lindane + 300 mg chlorobenzene/kg, were dosed with 1.05 g chlorobenzene + 18.3 mg lindane/kg. The glutathione analysis was conducted at this specific time since others have demonstrated that the nadir of hepatic glutathione was reached about 5 hr after administration of a challenge dose of the related chemical bromobenzene (2).

Analysis of variance for a 2 × 2-factorial experiment (25), Duncan's multiple range test (26), and a multiple logit analysis of variance (27) were used as aids in the interpretation of the data from this study.

RESULTS

In the first experiment, there were no significant differences in body weight, liver weight, kidney weight, or lung weight between the treated animals. Although there were no significant pretreatment effects on the storage of radioactivity in lung or adipose tissue (Table 1), pretreatment with both chlorobenzene and lindane significantly reduced the level of radioactivity in the liver 48 hr after the challenge dose of chlorobenzene was administered. The effect of pretreatment on the excretion of radioactivity in urine, feces, and expired air is presented in Table 2. While there was no pretreatment effect on the excretion of radioactivity in feces or expired air, both the chlorobenzene and lindane pretreatments significantly increased urinary radioactivity (Table 2). Furthermore, both these pretreatments significantly affected the distribution of radioactivity in the urine (Table 3). Both the lindane and chlorobenzene pretreatments significantly increased radioactivity in the glucuronide and polar fractions and this effect appeared to be additive in the rats pretreated with chlorobenzene + lindane (group AB).

Excretion of the chlorobenzene metabolites *o*-, *p*-, and *m*-chlorophenol were mon-

TABLE 1

Effect of Pretreatment on Tissue Radioactivity Content[a]

Pretreatment[b]		Treatment Group	Average tissue level of radioactivity		
Chlorobenzene	Lindane		Liver	Lung	Adipose tissue
0	0	C	18.1 ± 2.66	6.40 ± 1.43	0.056 ± 0.018
0	20	B	8.44 ± 2.33	6.09 ± 1.27	0.038 ± 0.013
300	0	A	9.29 ± 1.22	4.48 ± 0.761	0.048 ± 0.004
300	20	AB	5.26 ± 0.985	5.20 ± 2.03	0.019 ± 0.004

Analysis of variance

Source	d.f.	Mean square	Mean square	Mean square
A main effect	1	215[c]	11.9	0.0010
B main effect	1	281[c]	0.252	0.0030
A × B interaction	1	47.5	1.59	0.0001
Error	20	22.4	12.5	0.0010
Total	23	43.2	11.5	0.0009

[a] Values are mean % administered dose/g tissue × 10^3 ± SE.
[b] Treatment is described under Methods.
[c] $P < 0.01$.

TABLE 2
Effect of Pretreatment on Excretion of Radioactivity[a]

Pretreatment[b]		Treatment Group	Average excretion/24 hr		
Chlorobenzene	Lindane		Urine	Feces	Expired air
0	0	C	19.1 ± 1.18	0.97 ± 0.36	35.8 ± 8.92
0	20	B	22.0 ± 1.73	1.01 ± 0.44	38.4 ± 5.01
300	0	A	24.3 ± 1.92	1.63 ± 1.25	51.0 ± 4.92
300	20	AB	28.5 ± 1.65	0.85 ± 0.26	47.1 ± 8.41

Analysis of variance

Source	d.f.	Mean square	Mean square	Mean square
A main effect	1	208[c]	0.379	574
B main effect	1	77.4[c]	0.807	1.74
A × B interaction	1	2.47	1.02	41.5
Error	20	16.2	2.91	200
Total	23	26.6	2.63	201

[a] Values are mean % of administered dose ± SE.
[b] Treatment is described under Methods.
[c] $P < 0.05$.

itored in this study to determine the relative influence of the pretreatments on the three distinct metabolic pathways leading to their formation.

Accompanying a significant chlorobenzene main effect on the excretion of both conjugated and total chlorophenols was an interaction at $P = 0.07$ (Table 4). Snedecor

TABLE 3
Effect of Pretreatment on Distribution of Urinary Radioactivity[a]

Pretreatment[b]		Treatment Group	Average radioactivity in urine extract fractions			
Chloro-benzene	Lindane		Neutral Metabolites[c]	Glucuronides[d]	Sulfates[e]	Polar metabolites[f]
0	0	C	4.45 ± 0.461	6.44 ± 0.606	3.20 ± 0.427	3.73 ± 0.455
0	20	B	4.27 ± 0.730	7.35 ± 0.661	3.03 ± 0.523	5.21 ± 0.751
300	0	A	4.88 ± 0.572	8.36 ± 0.385	2.62 ± 0.236	5.12 ± 0.511
300	20	AB	5.25 ± 0.385	10.0 ± 0.623	3.47 ± 0.340	6.69 ± 0.571

Analysis of variance

Source	d.f.	Mean square	Mean square	Mean square	Mean square
A main	1	2.99	31.6[g]	0.030	12.4[h]
B main	1	0.050	9.95[h]	0.697	14.0[h]
A × B interaction	1	0.446	0.878	1.57	0.013
Error	20	1.30	2.01	0.941	2.04
Total	23	1.28	3.59	0.917	2.92

[a] Values are mean % of administered dose ± SE.
[b] Treatment is described under Methods.
[c] Radioactivity extracted with benzene from acid (pH = 2) urine.
[d] Radioactivity extracted with benzene after glucuronidase hydrolysis.
[e] Radioactivity extracted with benzene after sulfatase hydrolysis.
[f] Radioactivity remaining in residual urine after extraction of neutrals, glucuronides, and sulfates.
[g] $P < 0.01$.
[h] $P < 0.05$.

TABLE 4

Effect of Pretreatment on Conjugation of o-, p-, and m-Chlorophenol (CP)[a]

Pretreatment[b]		Treatment Group	Free and conjugated chlorophenol extretion/24 hr		
Chlorobenzene	Lindane		Total CP/24 hr	Free CP/24 hr	Conjugated CP/24 hr
0	0	C	9.19 ± 0.812	5.14 ± 0.570	4.05 ± 0.417
0	20	B	8.94 ± 0.842	5.60 ± 0.778	3.35 ± 0.492
300	0	A	10.9 ± 0.682	5.68 ± 0.377	5.24 ± 0.400
300	20	AB	14.8 ± 1.58	7.42 ± 1.18	7.38 ± 1.08
			Analysis of variance		
Source		d.f.	Mean square	Mean square	Mean square
A main effect		1	82.3[c]	7.96	38.8[c]
B main effect		1	19.0	6.94	2.94
A × B interaction		1	24.5[d]	2.33	11.5[d]
Error		19	6.48	3.56	2.63
Total		22	11.4	3.89	4.69

[a] Values are mean % of administered dose excreted/24 hr ± SE.
[b] Treatment is described under Methods.
[c] $P < 0.01$.
[d] $P < 0.10$.

and Cochran (25) recommend further comparison of the treatment groups in such cases. Consequently, the mean excretion of conjugated chlorophenols/24 hr and total chlorophenols/24 hr for groups A, B, AB, and C were compared by Duncan's multiple range test (26). The only significant ($P < 0.05$) comparison between the treatments indicated that group AB was greater than the other three groups. Thus, as a consequence of the interaction, the effect of pretreatment with chlorobenzene + lindane on chlorophenol excretion is greater than the additive effects of pretreatment with chlorobenzene and pretreatment with lindane.

The effect of pretreatment on the relative excretion of the chlorobenzene metabolites *o-*, *p-*, and *m*-chlorophenol is presented in Table 5. While there were no significant pretreatment effects on the excretion of *m*-chlorophenol, lindane pretreatment (group B) significantly elevated the urinary level of *o*-chlorophenol and significantly reduced that of *p*-chlorophenol while pretreatment with chlorobenzene (group A) had the opposite effect. The effect of pretreatment with the combination (group AB) appeared intermediate between that of group A and group B.

It has been suggested that glutathione depletion plays a role in the centrilobular necrosis induced by halogenated aromatic compounds (1, 4). Thus, hepatic glutathione content was determined 48 hr after administration of the challenge dose of chlorobenzene (Table 6). Results indicated that treatment with lindane produced a marginally significant increase ($P < 0.10$) in the hepatic level of glutathione. Hepatic glutathione was also examined in groups AB, C, and D 5 hr after a toxic dose of chlorobenzene was administered (Table 7). There was no significant difference in hepatic glutathione content between positive controls (group C) and rats pretreated with chlorobenzene + lindane (group AB). Both groups contained less than 50% of the normal (group D) hepatic glutathione level (Table 7).

A summary of the combined microscopic findings from the first and second experiments is presented in Table 8. In contrast to the normal histological appearance of the livers in rats of the negative control group (group D, Fig. 2), significant alteration, including centrilobular hepatocellular necrosis, was observed in the livers of most of the rats in three of the treated groups

TABLE 5
Effect of Pretreatment on Urinary o-, p-, and m-Chlorophenol[a]

Pretreatment[b]		Treatment Group	Relative excretion of chlorophenol metabolites (%)		
Chlorobenzene	Lindane		*o*-Chlorophenol	*p*-Chlorophenol	*m*-Chlorophenol
0	0	C	31.2 ± 0.706	44.1 ± 0.592	24.6 ± 0.626
0	20	B	33.7 ± 0.670	42.3 ± 0.406	23.9 ± 0.773
300	0	A	29.5 ± 0.774	46.2 ± 0.469	24.4 ± 0.537
300	20	AB	31.4 ± 0.835	43.8 ± 0.945	24.8 ± 1.20

Analysis of variance

Source	d.f.	Mean square	Mean square	Mean square
A main effect	1	24.1[d]	18.6[d]	0.453
B main effect	1	28.2[c]	24.9[c]	0.126
A × B interaction	1	0.585	0.302	1.93
Error	19	3.30	2.48	3.99
Total	22	5.12	4.07	3.55

[a] Values are mean (mg chlorophenol isomer × 100/total mg excreted chlorophenols) ± SE.
[b] Treatment is described under Methods.
[c] $P < 0.01$.
[d] $P < 0.05$.

(Table 8): the chlorobenzene-pretreated rats (group A), the lindane-pretreated rats (group B), and the positive controls (group C). The hepatocellular necrosis appeared to be most pronounced in group C (Fig. 3), somewhat less in group A, and generally of minimal or slight degree (although severe in one rat) in group B. In addition, in all three of these groups, the nonnecrotic liver cells in the central zones were slightly or moderately hypertrophied.

The necrotic cells were of two types: (i) those with deeply eosinophilic, contracted cytoplasm and nuclei that were pyknotic, lytic, fragmented, or absent; and (ii) greatly swollen cells with markedly vacuolated cy-

TABLE 6
Effects of Pretreatment on Hepatic Glutathione Content[a]

Pretreatment[b]		Treatment Group	Hepatic glutathione content (Average mg glutathione/g liver)
Chlorobenzene	Lindane		
0	0	C	1.88 ± 0.311
0	20	B	2.44 ± 0.251
300	0	A	2.40 ± 0.139
300	20	AB	2.64 ± 0.087

Analysis of variance

Source	d.f.	Mean square
A main effect	1	0.724
B main effect	1	0.937[c]
A × B interaction	1	0.149
Error	19	0.262
Total	22	0.312

[a] Values are mean mg glutathione/g liver, 48 hr after a toxic dose of chlorobenzene ± SE.
[b] Treatment is described under Methods.
[c] $P < 0.10$.

TABLE 7
Effect of Combined Treatment on Hepatic Glutathione Content[a]

Pretreatment[b]	Treatment group	Average mg glutathione/g liver	Percentage of normal GSH level
Negative control	D	3.27 ± 0.255[c]	100
Positive control	C	1.59 ± 0.130[d]	48.6
Chlorobenzene + Lindane	AB	1.38 ± 0.269[d]	42.2

[a] Values are mean mg glutathione/g liver, 5 hr after a toxic dose of chlorobenzene ± SE.
[b] Treatment is described under Methods.
[c–d] Values which are assigned the same superscript are not significantly different from one another ($P < 0.05$).

toplasm and pyknotic or absent nuclei. Stains for fat and glycogen did not disclose either of these substances in the cytoplasm of the swollen cells. The vacuolated appearance of these cells was thought to be consistent with hydropic change (increased inbibition of water). Varying proportions of leukocytes (chiefly histiocytes and occasional neutrophils) infiltrated the necrotic areas. The infiltration was most evident in rats of the C and A groups. However, in rats receiving both chlorobenzene and lindane pretreatment (group AB) there was absence of hepatocellular necrosis (Fig. 4) in all but one rat, in which only a minimal degree of necrosis was present. Slight to moderate hypertrophy of the liver cells was present in the central zones. When the incidence of lesions was tested in a multiple logit analysis of variance framework (27),

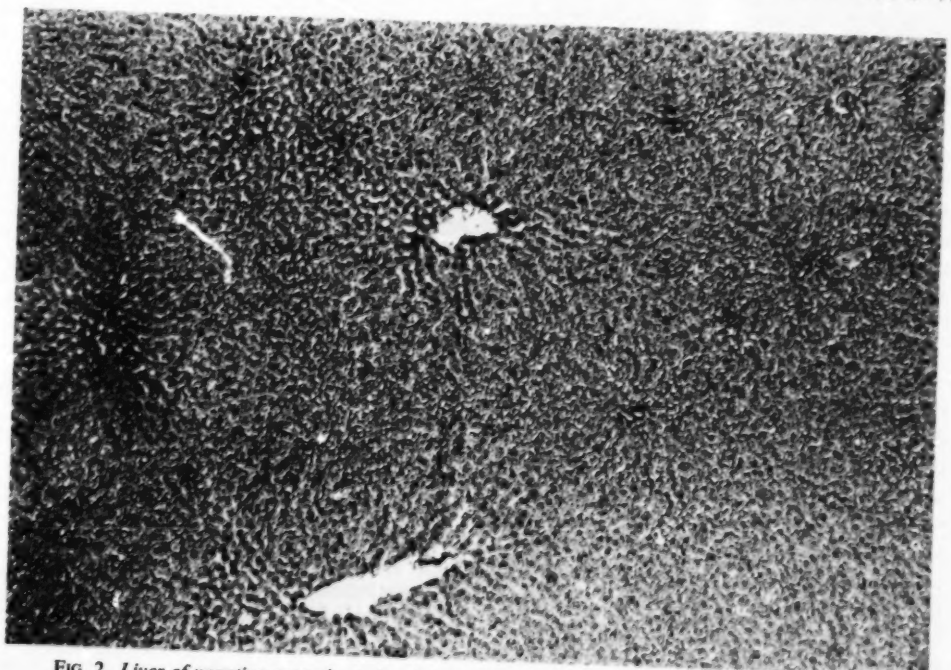

FIG. 2. *Liver of negative control rat showing no abnormalities. The two large spaces near midline are central veins. Hematoxylin and eoxin, ×35.*

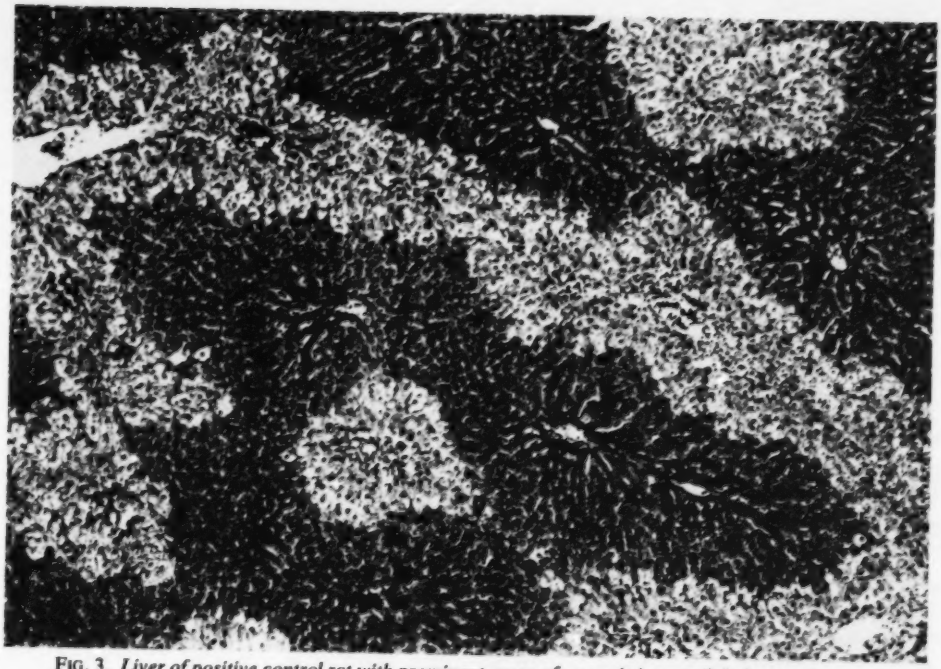

FIG. 3. *Liver of positive control rat with prominent areas of necrosis in centrilobular regions, some of which are confluent. Hematoxylin and eosin, ×35.*

there was a highly significant chlorobenzene × lindane interaction ($P = 0.03$) in terms of the approximate χ^2 statistic. However, when the degree of severity was taken into account (Table 9), pretreatment with lindane accounted for most of the observed antagonism to chlorobenzene-induced hepatotoxicity.

DISCUSSION

Previous workers have established that a threshold dose exists for halobenzene-induced hepatic necrosis (2, 4, 7). The dose threshold is due to depletion of hepatic glutathione levels (2, 4, 7) from the conversion of halobenzene epoxides to glutathione conjugates (2, 7). Once the formation of the chemically reactive 3,4-epoxide exceeds the synthesis of glutathione, covalent binding of this chemically reactive metabolite to tissue macromolecules takes place. Thus, the incidence and severity of chlorobenzene-induced hepatotoxicity are dependent on: (i) the proportion of the dose metabolized to chlorobenzene-3,4-epoxide; (ii) the rate at which the epoxide isomerizes to *p*-chlorophenol; (iii) the rate at which the epoxide conjugates with GSH; (iv) the rate at which the epoxide converts to a dihydrodiol by epoxide hydrase; and (v) the quantity of the epoxide which reacts with macromolecular nucleophiles. Pretreatment with chemicals that alter the relative importance of these factors are likely to influence chlorobenzene-induced hepatotoxicity.

Two complete replicates of a 2 × 2-factorial-designed experiment involving chlorobenzene and lindane were conducted to determine if pretreatment with either toxicant alone or in combination, could alter the metabolism and/or hepatotoxicity of chlorobenzene. The histopathological observations from each replicate were so similar that they were summarized in a single table (Table 8). From these observations it

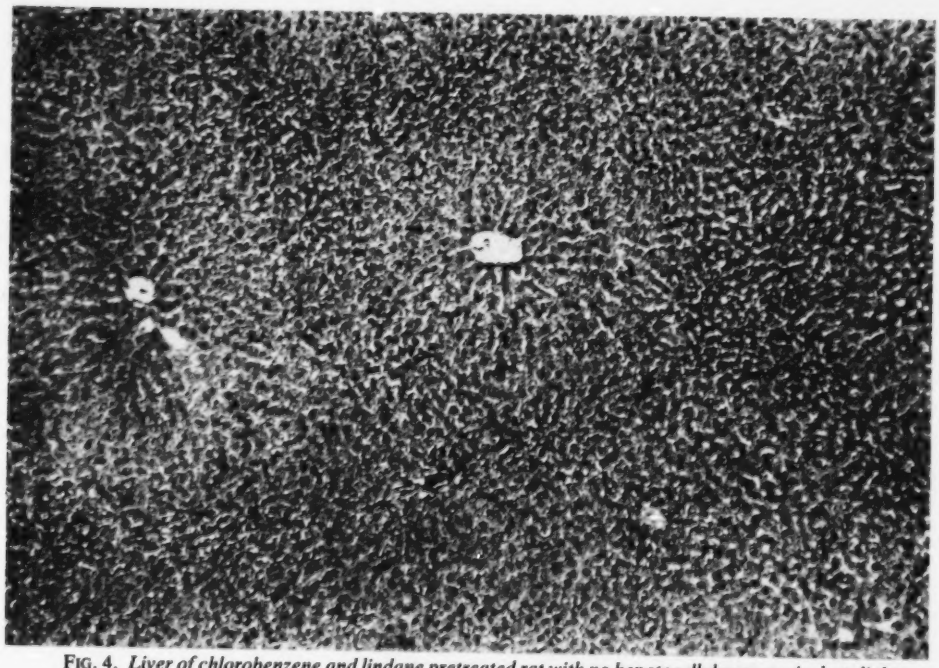

FIG. 4. *Liver of chlorobenzene and lindane pretreated rat with no hepatocellular necrosis, but slight hypertrophy of cells around central veins (the large spaces) is evident. Hematoxylin and eosin, ×35.*

was concluded that pretreatment with chlorobenzene (group A) had no effect on the incidence and a marginal effect ($P < 0.10$) on the severity of chlorobenzene-induced hepatotoxicity (Tables 8 and 9). In contrast, pretreatment with lindane (group B) significantly reduced both the incidence and the severity of the chlorobenzene-induced lesions. Finally, the coadministration of both lindane and chlorobenzene (group AB) for 7 days antagonized both the severity of chlorobenzene-induced hepatotoxicity and the incidence of lesions to the greatest extent observed. The incidence of lesions in group AB was significantly less than would be expected from the additive decreases due to pretreatment with chlorobenzene (group A) and lindane (group B).

Table 10 will be used to evaluate the comparative effect of pretreatment on the metabolism of chlorobenzene. Table 10 summarizes the relative changes in the pattern of chlorobenzene metabolism following pretreatment with lindane, chlorobenzene, or the combination of chlorobenzene and lindane.

Pretreatment with chlorobenzene (group A), while increasing metabolism to all three chlorophenol isomers, preferentially induced the pathway to *p*-chlorophenol to a greater extent (10%) than the paths to the other two isomers. Excreted *p*-chlorophenol should reflect the amount of chemically reactive chlorobenzene-3,4-epoxide that has not been detoxified by conjugation with GSH or by hydration. Thus this elevated level of the reactive toxic metabolite would be expected to increase hepatocellular necrosis, were it not for the 40% increase in metabolism to polar metabolites, glucuronides, and sulfates. Increased excretion of these metabolites represents inactivation of the toxic epoxide and thus there was a marginal reduction in hepato-

TABLE 8
Centrilobular Necrosis of Liver in Rats

Pretreatment Groups[a]	No. of rats examined histologically	Degree of severity of lesion[b]	No. of rats with lesion	Incidence of lesions
Chlorobenzene (Group A)	12	4+	1	91.7%
		3+	5	
		2+	2	
		1+	3	
Lindane (Group B)	11	4+	1	63.6%
		2+	1	
		1+	5	
Chlorobenzene + Lindane (Group AB)	12	1+	1	8.33%
Positive controls (Group C)	14	4+	4	85.7%
		3+	4	
		2+	3	
		1+	1	
Negative controls (Group D)	10			0%

[a] Treatment is described under Methods.
[b] 4+ = severe degree, 3+ = moderate degree, 2+ = slight degree, 1+ = minimal degree.

toxicity in rats pretreated with chlorobenzene despite the increased formation of chlorobenzene-3,4-epoxide.

Lindane pretreatment, on the other hand, increased the excretion of *o*-chlorophenol and decreased the excretion of the *p*-chlorophenol (Table 5). Moreover, there was a combined 66% increase in the excretion of

TABLE 9
Effect of Pretreatment on Centrilobular Hepatocellular Necrosis[a]

Pretreatment[b]		Treatment group	Severity of Lesion (mean degree of severity)
Chlorobenzene	Lindane		
0	0	C	2.50 ± 0.374
0	20	B	1.00 ± 0.357
300	0	A	2.17 ± 0.344
300	20	AB	0.083 ± 0.083

Analysis of variance

Source	d.f.	Mean square
A main effect	1	4.75[d]
B main effect	1	39.0[c]
A × B interaction	1	1.03
Error	45	1.25
Total	48	2.13

[a] Means (derived from data in Table 8) = (Σ Lesion score × no. of rats/score)/Total rats/treatment group ± SE.
[b] Treatment is described under Methods.
[c] $P < 0.01$.
[d] $P < 0.10$.

TABLE 10
Comparative Excretion of Chlorobenzene Metabolites[a]

Pretreatment[b] group	*o*-Chlorophenol[c]	*p*-Chlorophenol[c]	*m*-Chlorophenol[c]	Glucuronides + sulfates[d]	Polar metabolites[e]
C	100	100	100	100	100
B	105	93.3	94.4	126	140
A	113	124	117	103	137
AB	163	160	161	109	179

[a] Mean values of the positive controls (c) are represented as 100% and the mean values of the other rats relative to the control are presented as follows: % Excreted = (Mean % Adm. Dose of Pretreated Rats × 100)/(Mean % Adm. Dose of Positive Controls).
[b] Treatment is described under Methods.
[c] Values are derived from data in Tables 4–5.
[d] These values are calculated from the sum of the glucuronides + sulfates (Table 3) − conjugated chlorophenols (Table 4).
[e] Values are derived from data in Table 3.

glucuronides, sulfates, and polar metabolites. Thus, reduced formation of chlorobenzene-3,4-epoxide coupled with its enhanced inactivation could account for the significant reductions in both the incidence (Table 8) and severity (Table 9) of the lesions induced by chlorobenzene in these animals. In addition, it is possible that lindane pretreatment may enhance the recovery rate of hepatic glutathione since a marginally significant elevation in hepatic glutathione was observed 48 hr after administration of a challenge dose of chlorobenzene (Table 6).

Finally, it can be seen from Table 10 that pretreatment with chlorobenzene and lindane (group AB) produced a uniform 60% increase in the formation and excretion of all three chlorophenols. Accompanying this was a combined 88% increase in the excretion of polar and conjugated metabolites. The importance of these pathways, leading to the inactivation of chlorobenzene-3,4-epoxide, was increased by almost 30% relative to its formation. Thus, proportionately less of the chemically reactive chlorobenzene-3,4-epoxide was available for covalent binding to hepatic macromolecular nucleophiles. This together with additive increases in total urinary metabolite excretion (Table 2) provide the basis for the lack of toxicity that accompanied the accelerated metabolism in these animals. Significant reduction in the hepatic level of chlorobenzene-derived radioactivity in group AB supports this view (Table 1), since the reduced storage is consistent with the reduced toxicity found in these animals (Tables 8–9). In fact, the significant chlorobenzene and lindane interaction on the incidence of lesions (Table 8) indicates that the hepatic level of chlorobenzene-3,4-epoxide has been reduced below the threshold necessary to induce centrilobular hepatocellular necrosis.

It is suspected that unextracted chlorocatechols may constitute a significant portion of the polar metabolite fraction (Tables 3 and 10) for all three pretreatment groups (A, B, and AB). First of all, glutathione synthesis is thought to be rate-limiting in the covalent binding of the toxic epoxide (2, 4, 7), and since the positive controls already exhibit pronounced hepatocellular necrosis, hepatic glutathione levels in these animals should be depleted. Thus, a pretreatment-induced increase in polar metabolites would have to be due to either induced glutathione synthesis or to incomplete extraction of polar metabolites other than GSH conjugates. Since the hepatic glutathione level in chlorobenzene + lin-

dane pretreated rats was not significantly different from the positive controls 5 hr after the challenge dose of chlorobenzene, increased synthesis of glutathione appears unlikely. On the other hand, incomplete extraction of metabolites such as the chlorocatechols is a possibility since urine in these experiments was extracted with benzene (Table 3) whereas others have used diethyl ether (3). Finally, both lindane (28) and chlorobenzene (3) are known to induce epoxide hydrase and thus would be expected to increase the metabolism of chlorobenzene to chlorocatechols.

In conclusion, pretreatment of rats in two complete replicates of a 2 × 2-factorial-designed experiment, involving chlorobenzene and lindane, produced significant and rather selective changes in the various metabolic pathways of chlorobenzene that resulted in either (i) a marginally significant decrease in the chlorobenzene-induced hepatotoxicity (chlorobenzene pretreatment); (ii) significant reduction in both the incidence and severity of the lesions (lindane pretreatment); or (iii) absence of centrilobular hepatocellular necrosis in all but 1 of 12 rats where a minimal degree of necrosis was present (chlorobenzene + lindane pretreatment). It is clear from this study that the effect of pretreatment with xenobiotics on the hepatotoxicity of halobenzenes, such as chlorobenzene, depends on how the pretreatments affect the relative activity of all the enzymatic pathways involved and not just the formation of the chemically reactive metabolite.

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